

PLEIOTROPHIN GROWTH FACTOR RECEPTOR FOR THE TREATMENT OF PROLIFERATIVE, VASCULAR AND NEUROLOGICAL DISORDERS

Reference to Related Applications

5 This application claims priority to U.S. Provisional application number 60/211,491, filed June 14, 2000.

Rights in the Invention

10 This invention was made, in part, with United States government support under a grant from the United States Army, Medical Research Material Command, Breast Cancer Program, and also grant number CA58185, awarded by the National Cancer Institute, Specialized Program or Research Excellence (SPORE), and the United States Government has certain rights in the invention.

Background of the Invention

1. Field of the Invention

15 The present invention relates to the growth factor pleiotrophin, receptors for pleiotrophin, and fragments of these proteins, as well as nucleic acid sequences that encodes these proteins and protein fragments and antibodies reactive thereto. In particular, the invention relates to compositions and methods related to pleiotrophin and pleiotrophin receptor protein interactions for the treatment, prevention and detection of cell proliferation, vascular, neurological and developmental disorders, and also for the development of new compounds and methods therefor.

2. Description of the Background

20 Tumors of glial origin, including astrocytomas, oligodendrogliomas and ependymomas, account for almost eighty percent of all primary brain malignancies. Glioblastoma multiforme is both the single most common glial tumor and the most lethal, with a mean survival of only one year despite aggressive treatment (Holland, 2000). While these tumors exhibit multiple genetic alterations, including loss or mutation of the tumor suppressors PTEN (Wang et al., 1997), p53 (Albertoni et al., 1998) and INK4a-ARF (James et al., 1996), receptor tyrosine kinase (RTK) signaling seems to play a particularly important role in tumor development and growth. During the development of tumors, a continuous interaction between the malignantly transformed cancer cells
25 and the surrounding stromal cells takes place (Hanahan and Weinberg, 2000). During this cross-talk
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cancer cells induce other epithelial cells, immune cells, fibroblasts, endothelial cells, etc. to enhance the production of growth factors that will sustain the development of the tumor or, alternatively, to stop producing inhibitory factors that would impede further cancer cell expansion.

Along these lines, tumor cells will secrete growth factors that sustain their own growth by autocrine mechanisms and/or promote the growth of surrounding supportive cells and reduce their inhibitory signals by paracrine mechanisms. Glioblastomas and glioblastoma cell lines have been shown to overexpress the tyrosine kinase receptors for epidermal growth factor (EGF) (Nishikawa 1994), platelet-derived growth factor (PDGF) (Nister et al., 1991), hepatocyte growth factor (HGF) (Bowers et al., 2000), nerve growth factor (NGF) (Singer et al., 1999) and vascular endothelial growth factor (VEGF) (Holland et al., 2000). In addition, these tumors frequently overexpress the ligands for these RTKs, indicating a potential role for autocrine RTK signaling in glioblastoma growth. The importance of RTK signaling is also supported by the finding that the combined activation of two downstream targets of RTK signaling, Ras and Akt, in neural progenitor cells induces glioblastoma-like tumors in mice (Holland et al., 2000).

Summary of the Invention

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides new diagnostic, therapeutic and research tools, and methods relating to interactions between pleiotrophin and pleiotrophin receptors for the treatment and prevention of disorders. In particular, the invention relates to compositions and methods comprising pleiotrophin and pleiotrophin-receptor proteins, active fragments thereof and antibodies thereto, for stimulating or inhibiting cell proliferation.

One embodiment of the invention is directed to isolated polypeptide complexes comprising a pleiotrophin protein and a pleiotrophin-receptor protein. Pleiotrophin protein includes, but is not limited to, PTN, miple, midkine, recombinant pleiotrophin and combinations thereof. Pleiotrophin-receptor protein includes, but is not limited to, ALK, LTK, recombinant pleiotrophin-receptor protein and combinations thereof. Complexes may be physically bound to each other or unbound, and comprise whole proteins, protein fragments, recombinant proteins or fragments, synthetic proteins or fragments, proteins or fragment of human or non-human origin, or combinations thereof. Pleiotrophin-receptor protein fragments may lack a signal transduction activity such as, for example,

tyrosine kinase activity, and preferably comprise one or more, but not all portions of a full-length pleiotrophin receptor protein. The various portions of the pleiotrophin-receptor protein comprise an extracellular domain, an intracellular domain, a pleiotrophin binding site, a growth factor binding site, a mitogenic factor binding site, a tyrosine kinase, an antigenic domain, a heparin binding site, a glycosylated domain, a non-glycosylated domain, a signaling domain, a functional domain, a conserved domain, a transmembrane domain, and combinations thereof.

Another embodiment of the invention is directed to recombinant polypeptides comprising one or more, but not all regions of a full-length pleiotrophin receptor protein. The one or more regions include, but are not limited to an extracellular domain, an intracellular domain, a pleiotrophin binding site, a growth factor binding site, a mitogenic factor binding site, a tyrosine kinase, an antigenic domain, a heparin binding site, a glycosylated domain, a non-glycosylated domain, a signaling domain, a functional domain, a conserved domain, a transmembrane domain, and combinations thereof. Polypeptides are preferably antigenic and may have anti-angiogenic activity, induce apoptosis, or possess anti-motogenic, anti-mitogenic, cell proliferative or anti-cell proliferative activity.

Another embodiment of the invention is directed to nucleic acids that encode polypeptides of the invention. For example, nucleic acid may encode functionally or antigenically active polypeptides. Further, nucleic acids may be contained in vectors which may be further maintained in a host cell or organism.

Another embodiment of the invention is directed to compositions comprising polypeptides of the invention. Preferably, these polypeptides contain a pleiotrophin-binding site, and may be recombinant, synthetic or peptido-mimetics. Recombinant polypeptides may comprise one or more, but not all regions of a full-length pleiotrophin protein. Regions that may be included are a pleiotrophin receptor binding portion, which binds, but does not activate a pleiotrophin-receptor protein. Further embodiments include nucleic acids that encode these polypeptides, which may encode functionally or antigenically active polypeptides, vectors that contain these nucleic acids, and compositions comprising the polypeptides, which may further contain a pharmaceutically acceptable carrier such as, for example, water, oils, alcohols, salts, fatty acids, saccharides, polysaccharides and combinations thereof.

Another embodiment of the invention is directed to antibodies that are reactive against a pleiotrophin protein such as, for example, PTN, and also antibodies that are specifically reactive against pleiotrophin-receptor proteins such as, for example, ALK. Preferably, antibodies are reactive against the interacting sites of the pleiotrophin protein and the pleiotrophin-receptor protein, and may be of any isotype or fragment including, but not limited to, IgG, IgM, Fab fragments, Fv fragments, recombinant antibodies and humanized antibodies. Preferably the antibody is a monoclonal antibody and, thus, the invention is further directed to hybridomas that produce such monoclonal antibodies. A pleiotrophin binding site of the pleiotrophin-receptor protein PTN is preferably between amino acid sequence positions 368 and 447, with the actual contact site between amino acid sequence positions 391 and 401. Antibodies of the invention may block or stimulate activation of the pleiotrophin-receptor protein.

Another embodiment of the invention is directed to kits comprising a pleiotrophin-binding region of a pleiotrophin-receptor protein and a pleiotrophin-receptor binding region of a pleiotrophin protein, for screening substances for an ability to block interaction between pleiotrophin and pleiotrophin receptor. The pleiotrophin-binding region preferably comprises amino acid positions 391-401 of ALK, and substances which can be evaluated for their ability to interfere with interaction include, but are not limited to, antibodies, additional pleiotrophin proteins, additional pleiotrophin-receptor proteins, drugs, anti-angiogenic substances, anti-proliferative substances, anti-motogenic substances, anti-metastatic substances, apoptotic substances, anti-tumorigenic substances, anti-neoplastic substances, biologically active substances and combinations thereof.

Another embodiment of the invention is directed to methods for evaluating an activity of a substance to block interaction between pleiotrophin and pleiotrophin receptors comprising incubating an amount of the substance with at least interacting portions of a pleiotrophin protein and a pleiotrophin receptor protein, determining a first measure of interaction between the interacting portions, comparing the first measure of interaction with a second measure of interaction determined with a different amount of the substance, and evaluating the activity of the substance. Incubating may comprises contacting the substance with the interacting portions for a predetermined period of time and at a predetermined temperature. Preferably the predetermined period of time is between one second and ten minutes, but may be longer or shorter as desired, and the predetermined

temperature is between 4°C and 37°C, but may be warmer or cooler, as desired. The amount of substance to be tested is preferably a physiologically effective amount, but may be any amount including, but not limited to, no substance, ten percent of a physiologically effective amount, twenty percent of a physiologically effective amount, fifty percent of a physiologically effective amount, a physiologically effective amount, two times a physiologically effective amount and ten times a physiologically effective amount. Substance that can be evaluated include, but are not limited to, antibodies, additional pleiotrophin proteins, additional pleiotrophin-receptor proteins, drugs, anti-angiogenic substances, anti-proliferative and proliferative substances, anti-motogenic and motogenic substances, anti-metastatic substances, apoptotic substances, anti-tumorigenic substances, anti-neoplastic substances, biologically active substances and combinations thereof. Further, activities that can be tested include, but are not limited to, anti-angiogenic activity, anti-proliferative and proliferative activity, anti-motogenic and motogenic activity, anti-metastatic activity, apoptotic activity, anti-tumorigenic activity, anti-neoplastic activity, and combinations thereof. A further embodiment includes a method for evaluating an activity of a substance comprising incubating the substance with at least interacting portions of a pleiotrophin protein and a pleiotrophin receptor protein for a first period of time, determining a first measure of interaction between the interacting portions, incubating the substance with the at least the interacting portions for a second period of time, determining a second measure of interaction between the interacting portions, comparing the first measure of interaction with the second measure of interaction, and evaluating the activity of the substance.

Another embodiment of the invention is directed to kits for the detection of cell proliferative diseases such as, for example, cancer and heart disease. Kits comprise all or characteristic portions of pleiotrophin and/or pleiotrophin receptor proteins, or antibodies to these proteins and/or polypeptides.

Another embodiment of the invention is directed to methods for treating a patient, preferably a human, comprising administering to said patient a therapeutically effective dose of a composition comprising a pleiotrophin-receptor protein or fragment thereof. Preferably, administering comprises direct injection of the composition, and the therapeutically effective dose is that amount which will bind to at least half of free pleiotrophin of said patient. Fragments preferably comprise a

pleiotrophin-binding portion of the pleiotrophin-receptor protein. Preferably, treatment stimulates a cell proliferation such as creation and growth of blood vessels, or prevents a cell proliferation such as a tumor or a metastasis. A further embodiment of the invention is directed to methods for blocking or stimulating tyrosine kinase activity, and thereby stimulating an anti-neoplastic effect, by interfering with the binding of pleiotrophin with pleiotrophin receptor. Interference with binding and/or interaction can be performed by administering to patients compositions containing modifications and/or derivatives of pleiotrophin, antibodies to pleiotrophin-binding sites or other compounds that block receptor activation. Compositions of the invention may be administered in a therapeutically safe and effective dose to humans and other mammals in the form of pills, tablets, powder, liquid or combinations thereof.

Another embodiment of the invention is directed to compositions and methods comprising pleiotrophin, pleiotrophin-receptor, and modifications and derivatives of either that are useful as pharmaceuticals in the treatment or prophylaxis of diseases such as cell proliferative disorders including cancer and heart disease. Compositions may be useful in therapy, prophylaxis, diagnosis, or as research tools, and may further comprise pharmaceutically acceptable carriers for use in the treatment or prevention of diseases and disorders. A further embodiment is directed to compositions comprising nucleic acid sequences that contain the anti-sense of pleiotrophin or pleiotrophin-receptor genes or representative portions thereof. Sequences may be useful in compositions for the treatment of breast and other neoplastic disorders by reducing or shutting down pleiotrophin receptor expression in cells.

Other embodiments and advantages of the invention are set forth in part in the description which follows, and in part, will be obvious from this description, or may be learned from the practice of the invention.

Description of the Figures

Figure 1 (a) Schematic of ALK gene showing factor binding sites. (b) Binding of the PTN to recombinant ECD of ALK. (c) SELDI analysis of PTN-ligand binding. (d) SELDI analysis of conditioned media, initial PTN binding to ECD, and completed PTN binding to ECD.

Figure 2 (a) Competition of PTN binding to 32D/ALK-transfected cells, and (b) saturation binding of PTN to 32D/ALK and 32D/control cells.

Figure 3 (a) SW-13 or SW-13/ALK cells stimulated with PTN and immunoprecipitated with anti-PY, anti-ALK, or anti-IRS-1 antibodies. (b) SW-13/ALK cells stimulated with PTN, ECD or anti-PTN and immunoprecipitated with anti-PY or WB anti-PTN. (c) PTN stimulation of SW-13/ALK cells immunoprecipitated with anti-PY.

5 Figure 4 Affect of ALK overexpression on PTN-stimulated growth.

Figure 5 (a-d) ALK expression in human GBM tissue and cell lines. (e) Detection of ALK mRNA in various cell lines by RNase protection.

Figure 6 (a-c) Pleiotrophin-induced AKT phosphorylation in U87MG cells.

Figure 7 (a) Autoradiogram and (b) quantitation by phosphoimager analysis of ALK

10 mRNA. (c) Dose response of pleiotrophin, and (d) PDGF BB on phosphoAkt/Akt. (e)

Comparison of the effect of PTN as a function of ALK levels in different cell lines.

Figure 8 Xenograft tumor growth showing (a) size and (b) relative ALK/GAPDH as a function of time after tumor cell inoculation. (c) Mice survival curves from panel (a).

Figure 9 Analysis of mitosis and apoptosis in tumor xenografts using (a) a high power (400 X) H & E (left column) and TUNEL (right column) stained sections from size-matched pRC/CMV (upper row) and Rz1-2 (lower row) xenograft tumors. (b) Number of mitotic figures and TUNEL positive cells as a function of relative ALK levels.

Description of the Invention

As embodied and broadly described herein, the present invention is directed to nucleic acid sequences encoding pleiotrophin and pleiotrophin-receptor proteins, to complexes of these proteins and protein fragments, to pharmaceutical compositions comprising proteins, complexes and fragments thereof, to antibodies to these proteins and polypeptides, to kits and methods that relate to interaction between pleiotrophin and pleiotrophin receptor, and to compositions and methods for the treatment, prevention and detection of disease and, in particular, neoplastic disease in patients.

25 Pleiotrophin is a heparin-binding growth factor that has been implicated in tumor growth, cellular invasion of tissues (i.e. motogenic), angiogenesis and metastasis. As used herein, the term pleiotrophin refers to a family of proteins that bind to a pleiotrophin receptor and share a similar conserved sequence. Members of this family include isoforms thereof, and have been identified in the human (PTN; midkine), the rat, the mouse (PTN, midkine), the chicken, the fruit fly *Drosophila*

(miple), and many other species, and is believed present in most all eukaryotic species including yeast and insects. Pleiotrophin is expressed in a developmentally regulated manner in rat brain (Li et al., 1990; Merenmies and Rauvala, 1990), and also promotes neurite outgrowth activity. This growth factor is highly expressed in the developing nervous system, but down-regulated in adults.

5 Further, pleiotrophin is secreted by human cancer cell lines (Wellstein et al., 1992), and has mitogenic activity on fibroblast, epithelial and endothelial cells and, thus, contributes to the metastatic phenotype as a tumor growth factor and angiogenic factor (Schulte and Wellstein, 1997). Serum samples from cancer patients contain elevated levels of pleiotrophin that can stimulate cells both in an autocrine as well as a paracrine manner (Schulte and Wellstein, 1998; Souttou et al., 10 1998). In addition, pleiotrophin targeting by ribozymes (Czubayko et al., 1997a) or by transfection of mutant pleiotrophin cDNA that leads to the formation of inactive dimers (Zhang et al., 1997), inhibited human cancer cell growth in animal models.

Relative to normal brain, pleiotrophin expression is elevated following acute ischemic injury (Yeh et al., 1998), and also in tumors (Schulte et al., 1997), thus implicating pleiotrophin as a tumor growth factor insofar as the reactivation of a developmentally-regulated signaling pathway may provide a tumor with a powerful growth signal. And, in fact, pleiotrophin expression induces tumor growth and metastasis of NIH3T3 cells (Chauhan et al., 1993), and has a rate-limiting role both as an angiogenic factor (Choudhuri et al., 1993) and a tumor growth factor for different tumors including melanoma and choriocarcinoma (Czubayko et al., 1994; Czubayko et al., 1996; Schulte et al., 1996; Schulte et al., 1997). In addition, pleiotrophin activates both the Ras-MAPK and the PI3K-Akt signaling axes (Souttou et al., 1997), both pathways implicated in glial tumorigenesis (Holland et al., 2000). In view of these functions, dysregulation of pleiotrophin would have substantial implications for the treatment of diseases such as neurodegenerative disease and cell proliferative disorders such as cancer.

25 It was surprisingly discovered that, ALK, an orphan RTK, is a cell receptor for pleiotrophin in humans. As a previously unknown target for the ligand, this receptor provides a mechanism to regulate physiological functions attributed to pleiotrophin, which include, but are not limited to, prevention and treatment of cell proliferative disorders such as cancer, induction of neurite outgrowth, maintenance of neuronal function, maintenance of developmental regulation, promotion

of cell proliferation for wound healing and, in particular, endothelial cell proliferation such as, for example, vessel and microvessel growth and formation in cardiac tissues, in both central and peripheral neural tissues, and any endothelial cells of the body. The pleiotrophin activity, ALK tyrosine kinase, was originally identified as a fusion protein with nucleophosmin (npm/ALK) due to a t(2;5) translocation (Morris et al., 1994). This fusion results in constitutive activation of the intracellular ALK kinase and was shown to induce anaplastic lymphoma. The full-length ALK receptor has been shown to be highly expressed in the developing nervous system and down-regulated postnatally (Iwahara et al., 1997), very similar to the expression profile of its ligand, pleiotrophin. As shown in Table 1, ALK expression (or the lack thereof) correlated with the growth effect of pleiotrophin on cells tested. It has also been surprisingly discovered that this tyrosine kinase receptor is over expressed in human glioblastoma and is rate-limiting for the growth of a xenograft model of glioblastoma. The term pleiotrophin-receptor protein, refers to a family of proteins, including isoforms, that are involved in pleiotrophin stimulation or signaling. Members of this family have been identified in humans, rats, mice, chickens, Drosophila, and many other species, and is believed present in most all eukaryotic species including yeast and insects. Although all have the common feature in that they are involved in pleiotrophin stimulation, some, like ALK (mouse and human), actually bind to pleiotrophin while others, like LTK (Drosophila), act as co-signaling proteins or co-receptor proteins that are involved in the down-stream signaling (i.e. signal transduction) that results from pleiotrophin binding.

Accordingly, one embodiment of the invention is directed to isolated complexes comprising pleiotrophin protein and pleiotrophin-receptor protein. These complexes may comprise all or specific portions of pleiotrophin and/or pleiotrophin-receptor proteins. Complexes may be physically bound (covalently or non-covalently) or unbound. The human pleiotrophin receptor, ALK, is composed of three principal regions, an extracellular domain (ECD), a transmembrane region (TM), and an intracellular domain (ICD) (see Figure 1a). Within the ECD is the pleiotrophin-binding site (amino acids 368-447 with actual contacts at amino acids 391-401) which, upon binding with pleiotrophin, induces pleiotrophin-associated functions including cell proliferation such as tumor growth, endothelial cell growth and neural cell growth, activation of Ras-related activities, cellular invasion (motogenic activities) such as metastasis, angiogenic activities, and developmental

regulation to name a few. Within the ECD are also the MAM domains, typical signature sequence patterns for ECDs. MAM domains are also found in *Drosophila* ALK protein which contains the PTN binding site and has an overall 42% similarity with the human ALK protein. In contrast, LTK, the closest homolog of ALK is a 100-kDa transmembrane protein with a short ECD that lacks 60% of the N-terminal portion of the ALK ECD and the PTN binding domain. The MAM domains of ALK, one of which encompasses the pleiotrophin-binding site, are found in the ECDs of a diverse family of transmembrane proteins (Protein Database PDOC 00604). The ICD contains the tyrosine kinase activity and the translocation site in the juxtamembrane region for fusion with nucleophosmin (t(2,5) NPM-ALK). The ICD also contain consensus binding sites for insulin receptor substrate-1 (IRS-1), and binding sites for the factors Shc and PLC-gamma. The protein is also believed to be glycosylated and contain one or more heparin-binding sites. Pleiotrophin and pleiotrophin-receptor proteins such as human and mouse ALK, and *Drosophila* LTK have been cloned and their gene sequences may be recombined to express only one or more of the nucleic acid or amino acid domains (e.g. exons, introns, conserved amino acid or nucleic acid regions, open reading frames in any of the three possible reading frames, binding sites for pleiotrophin, mitogenic factors, growth factors, mitogenic factors, insulin and heparin, signaling domains, glycosylated and non-glycosylated regions, functional domains, co-receptors, co-signaling factors, and consensus sequences of the pleiotrophin-receptor proteins). For example, factors containing only a pleiotrophin-binding portion and not a signaling or tyrosine kinase portion may be useful to bind pleiotrophin and, thereby, effectively remove pleiotrophin stimulation from a disease pathway. These blocking or interfering peptides may be free in solution (e.g. aqueous or non-aqueous such as oil-based), or contain a transmembrane portion (of ALK or another appropriate portion) to fix such recombinant molecules in cell or vesicle walls to more effectively or efficiently bind pleiotrophin. Alternatively, the tyrosine kinase activity or other signaling activity may be engineered out of pleiotrophin-receptor proteins such that pleiotrophin binding would not have the undesirable effect such as a mitogenic, motogenic or cell proliferative effect, but would have one or more positive activities such as induction of apoptosis, or an anti-proliferative or anti-metastatic effects. Further still, one or more other binding sites may be engineered into the pleiotrophin receptor protein such that pleiotrophin binding would induce a cascade of beneficial functions to a host to fight disease or maintain proper development.

Homologs and derivatives of these protein fragments may be created by those of ordinary skill in the art. Homologs are peptides that are functional equivalents to the peptides of the invention, but contain differences such as, for example, one or more of the amino acid substitutions including, but not limited to, one or more conservative amino acid substitutions. Proteins and protein fragments including isoforms, recombinant proteins, homologs, derivatives (e.g. modified proteins and homologs such as glycosylation changes) and bound complexes may be isolated from one or more other components, or purified to homogeneity or near homogeneity, as desired.

Procedures to isolate and purify pleiotrophin and pleiotrophin-receptor protein, protein fragments, recombinant proteins and complexes thereof are well known to those of ordinary skill in the art. Alternatively, proteins and polypeptides of the invention can be recombinantly or synthetically produced. Recombinant polypeptides can be created by manipulating the nucleic acid sequences of the invention removing undesired portion and/or adding other desired portions. Synthetic polypeptides of the invention can be created by replacing amino acid sequences of the polypeptides with non-natural amino acids or substituting the natural L forms of one or more amino acids to D-amino acids. In this way, polypeptides of the invention can be created that are resistant to proteases that may be found in a body. Further, peptido-mimetic polypeptides can be created which mimic the three-dimensional structure of the polypeptide, or portion thereof, for purposes of evaluating structure-function relationships. All these procedures are well-known to those of ordinary skill in the art.

Another embodiment of the invention is directed to nucleic acids that comprises all or one or more portions of the sequence of a pleiotrophin receptor, to peptides derived from these sequences, and to sequences complementary thereto. The nucleic acid sequence that encodes the pleiotrophin receptor, ALK, can be found in GenBank at accession number U66559. Nucleic acids of the invention may be single-stranded or double-stranded and composed of DNA, RNA or PNA, or another appropriate nucleic acid, polypeptide or functionally similar backbone structure. Single stranded nucleic acids may be in the form of a sense strand or an anti-sense strand. Further, receptor genes of the invention may be derived from other mammals besides humans (using identification and isolation procedures and techniques that are well known to those of ordinary skill in the art) such as, for example, mice, rats or any rodent, mammals such as cattle, sheep, goats, pigs, horses, canines and

felines, and most any other animal. Receptor genes of the invention include other nucleic acid sequences that may be identified, using techniques and procedures well known to those of ordinary skill in the art, that are found in insects (*Drosophila*), plants, yeast and other eukaryotic organisms.

Nucleic acids according to the invention include isolated (e.g. semi-purified and purified to homogeneity) and recombinant nucleic acid sequences comprising all or one or more active fragments of the pleiotrophin-receptor polypeptide. Nucleic acids containing conserved regions of sequences and nucleic acids encoding open reading frames and conserved domains within open reading frames are typically sufficient to represent or contain identifiable portions of pleiotrophin receptor such as functional and antigenic portions. Nucleic acids may comprise additional sequences such as pleiotrophin receptor-specific promoters, activator and repressor sites, and enhancers for modulation of expression of sense or antisense messages, recombination sequences for gene targeting, selectable markers for transfections, or replication origins for passage in a host such as bacteria or any prokaryotic cells, virus, eukaryotic cells or yeast. Thus, the invention includes recombinant cells containing nucleic acids of the invention. A further embodiment of the invention includes pleiotrophin receptor-specific promoters which modulation transcription of pleiotrophin receptor in normal, pre-malignant and malignant cell. These promoters can be functionally coupled to anti-neoplastic genes to diagnose, identify, treat or prevent cell proliferative disorders such as, for example, tumors, breast carcinoma, prostate cancer, and metastatic disease, development disorders and neurological disorders, and also endothelial disorders in which cell proliferation is desired. Nucleic acids may be packaged in a viral vector such as, for example, a retroviral, a vaccinia or an adenoviral vector. In one embodiment, the sequence may be part of a homologous recombination vector designed to recombine with another sequence. The invention further includes vectors comprising the nucleic acid sequences of the invention, polypeptides expressed by these vectors, and recombinant cells comprising these vectors.

Nucleic acids and polypeptides or proteins according to the invention may be used as a diagnostic or therapeutic tool in the detection, treatment or prevention of diseases, such as neoplastic disorders (e.g. malignant tumors, metastatic disease), developmental disorders, neuronal cell disorders, and cell or tissue growth disorders. For example, one embodiment of the invention is directed to diagnostic aids or kits for the detection of disorders in a patient. Detection kits may

comprise pleiotrophin-receptor nucleic acid sequences or pleiotrophin-receptor polypeptides whose presence or absence in the sample would be indicative of the presence of a disease such as, for example, breast cancer or a metastasis. As shown in Table 1, there is a clear correlation of expression of ALK with PTN response. Further, as there was no detectable ALK mRNA in hematopoietic cells, side effects attributable to therapeutics of the invention, or developed with the invention, are expected to be minimal. Samples which can be analyzed include samples of biological fluids (e.g. blood, plasma, interstitial fluid, urine, cerebrospinal fluid) and samples of biological tissue (e.g. surgical biopsy). Another embodiment is directed to compositions and method for the treatment of patients that induce cell proliferation and, in particular, endothelial cell proliferation.

Endothelial cells are found throughout the body and in most every organ including the brain, heart and peripheral nervous system. After an ischemic attack or any restriction of blood flow or circulation, there is typically damage to effected regions or organs of the body such as, for example, the heart, retina or a limb (such as occurs in diabetics). Compositions of the invention can be used to stimulate endothelial cell proliferation and specifically the proliferation and generation of new blood vessels to effected areas.

Another embodiment of the invention is directed to antibodies specifically reactive against polypeptides and proteins and fragments thereof of the invention. Antibodies may be polyclonal or monoclonal of any isotype (e.g. IgA, IgD, IgE, IgG1, IgG2a, IgG2b, or IgM), or antibody fragments (e.g. Fab, Fv), humanized antibodies, or recombinant or synthetic antibodies (e.g. variable region fragments). Further, the invention comprises hybridomas that express antibodies specifically reactive against proteins and polypeptides of the invention. Preferably, antibodies are specifically reactive against the interacting portions of pleiotrophin and the pleiotrophin receptor. Antibodies have been created which block binding between the two proteins. These antibodies were created to be reactive against amino acid sequence 368-447 of the receptor protein, which is believed to be the region directly involved in protein-protein interaction. Antibodies that interfere and negatively effect pleiotrophin-receptor protein activation as well as antibodies that positively stimulate pleiotrophin-receptor protein activation have been developed. Both are useful in methods of the invention. For example, such antibodies (i.e. antibodies that interfere with pleiotrophin binding and/or interact with receptor at the pleiotrophin-binding site) that negatively stimulate the receptor protein are useful as

therapeutics in the treatment of tumors and metastatic disease. Such antibodies that positively stimulate the receptor protein are also useful to induce cell proliferation when, for example, the proliferation of blood vessels and microvessels is desired after a stroke or heart attack, or to prevent limb, organ or tissue degeneration in diabetics.

Another embodiment of the invention is directed to kits and methods which can be used to screen various substances for the ability to effect pleiotrophin-pleiotrophinreceptor interaction and/or pleiotrophin receptor signaling. Kits may comprise the interacting portions of the pleiotrophin and pleiotrophin receptor proteins. Preferably, the portion of the pleiotrophin-receptor protein comprises the pleiotrophin-binding region such as, amino acids 368-447 or 391-401 of PTN. Basically, substances to be examined are incubated in the kits with the interacting portions. Incubations may be for a predetermine time and at a pre-determined temperature. Preferably, times are between one second and ten hours, preferably between one minute and one hour, and longer for incubations with cells such as between one and thirty days, preferably between two and ten days, but may be longer or shorter for each as desired or as the materials require. Temperatures are preferably between 4°C and 37°C, but may be warmer or cooler as desired. More preferably, incubations are conducted at room temperatures (e.g. between 19°C and 25°C). Using the peptides of the invention, incubations can be varied to evaluate the effects of exposure time on the interaction of the substance. This evaluation is not possible using proteins that are constitutively turned on (i.e. induce signaling effects as if continually bound with pleiotrophin). Substances that can be evaluated include most any substance such as, for example, antibodies, different pleiotrophin proteins, different pleiotrophin-receptor proteins, drugs, anti-angiogenic substances, anti-proliferative and proliferative substances, anti-motogenic and motogenic substances, anti-metastatic substances, apoptotic substances, anti-tumorigenic substances, anti-neoplastic substances, biologically active substances and combinations thereof. Activities that can be examined or tested include, for example, anti-angiogenic activity, anti-proliferative activity, anti-motogenic activity, anti-metastatic activity, apoptotic activity, anti-tumorigenic activity, anti-neoplastic activity, and combinations thereof.

Another embodiment of the invention is directed to a method for treating a disorder comprising administering a therapeutically effective amount of a composition comprising pleiotrophin-receptor proteins, fragments or ligands of these proteins and fragments. Such

compositions may be anti-angiogenic, induce apoptosis, induce cell proliferation (e.g. endothelial and blood vessel growth), and/or stimulate a cell or humoral response. For example, in cell growth disorders such as breast cancer and other neoplasias, cytokine expression may be improperly turned off (e.g. methylated) in malignant or pre-malignant cells. As such, these gene products may also be useful as a diagnostic for malignancy. A therapeutically effective dose in a mammal is that amount of protein or protein fragment that will bind at least half, and preferably at least 70% and more preferably at least 90% of the available pleiotrophin of a patient. Alternatively, compositions of the invention may be useful in, for example, neurological or developmental disorders to turn on genes whose activity may be improperly reduced or turned off. Further, polypeptides of the invention may induce apoptosis and are useful as therapeutics to treat and prevent neoplasia such as, for example, tumors, metastasis and any uncontrolled cell growth. A further embodiment of the invention comprises the pleiotrophin-receptor promoters which may be differentially modulated and thereby regulate expression of a desired gene. Alternatively, pleiotrophin-receptor protein, or effective portions thereof, may be useful to treat patients by binding available pleiotrophin in a system and thereby preventing pleiotrophin stimulation.

The peptide, polypeptide, protein or fragment thereof, or ligand thereto, may be administered by injection, orally, pulmonary absorption, topical application or delayed release, as desired. Although parenteral administration would be preferred, administration of pleiotrophin receptor would preferably be by injection directly to the tumor, tissue and/or cells to be treated as unprotected proteins would be broken down in the gut or after prolonged exposure to a patient system. The composition may further comprise a pharmaceutically acceptable carrier such as water, alcohols, salts, oils, glycerols, fatty acids, starches, saccharides, polysaccharides or combinations thereof. More than one carrier may be used together to create a pharmaceutical with desirable properties such as delayed release, protection from the harsh gut environment and the like. A further embodiment of the invention comprises vaccines for the treatment and/or prevention of cell proliferative disorders such as neoplastic disease and also diabetes (where growth of blood vessels in the body is desired). Vaccines may comprise antibodies reactive against polypeptides and/or polypeptide fragments of the invention, and the polypeptides and/or fragments themselves. Vaccines comprise therapeutically

effective doses of the therapeutic agent, which may be the polypeptide or polypeptide fragment, or an antibody or collection of antibodies which bind or are otherwise reactive thereto.

Another embodiment of the invention is directed to nucleic acids therapies derived or based on the sequence of pleiotrophin receptor (e.g. ALK), useful in diagnosis and in diagnostic kits.

5 Therapy may involve using the sequences, or effective parts thereof, in gene therapy, including gene ablation, gene expression and gene suppression, such as anti-sense suppression. Diagnosis may involve genotypic analysis of samples to determine the existence and expression levels of the genes.

Nucleic acids of the present invention may be used in various treatment and research modalities, including gene replacement, gene targeting, anti-sense inhibition, anti-sense blocking, genetic ablation and gene silencing. Gene replacement involves replacing a copy of a defective gene with another copy by homologous recombination. Gene targeting involves the disruption of a cellular copy of a gene by homologous recombination. Gene targeting refers to a process of introducing a nucleic acid construct into a cell to specifically recombine with a target gene in the cell. The nucleic acid construct inactivates the gene after targeting. Inactivation may be by introduction of termination codons into a coding region or introduction of a repression site into a regulatory sequence. Anti-sense inhibition exploits the specificity of hybridization reactions between two complementary nucleic acid chains to suppress gene expression. If a cloned gene is engineered so that only the opposite DNA strand is transcribed, the resultant RNA may hybridize to the sense RNA and inhibit gene expression. Anti-sense blocking refers to the incorporation into a cell of expression sequences

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which direct the synthesis of anti-sense RNA to block expression of a target gene. Anti-sense RNA hybridizes to the mRNA of the target gene to inhibit expression. Genetic ablation (gene knockout) refers to one process of silencing a gene in a cell. Genetic ablation (gene knockout) may be performed after a cell is selected for use or by selecting a cell already comprising a genotype with the proper genetic ablation. Ablation of the gene encoding pleiotrophin-receptor protein, for example by pre-transcriptional inhibition (such as homologous recombination with endogenous recessive oncogenes) or post transcriptional inhibition (such as the expression of anti-sense oncogenes to suppress translation) may be useful. Gene silencing is performed by transfecting cells with nucleic acids which cause genetic ablation or by anti-sense suppression. The silencing process may include processes such as gene targeting or anti-sense blocking.

Another embodiment of the invention is directed to methods of blocking of pleiotrophin-mediated activities. Blocking involves interfering with pleiotrophin binding and/or activation of the pleiotrophin receptor and, as a consequence, reducing or eliminating pleiotrophin-mediated activities such as, for example, tyrosine kinase activity of the receptor protein. Blocking receptor protein activity can play a significant role in development and a therapeutic role in disease processes by, for example, inducing for example, apoptosis, anti-angiogenesis, maintaining the regulation of development, maintaining neuronal function, preventing the activation of Ras and other oncogene pathways, reducing invasion by transformed cells of tissues and organs, preventing or reducing metastatic induction, and depriving transformed cells and tumors of access to growth factors, angiogenic factors, mitogenic factors. Blocking may be performed by administering proteins or protein fragments of the invention which retain the ability to bind pleiotrophin, but not activate pleiotrophin-receptor pathways, or prevent tyrosine kinase activity of the pleiotrophin receptor. Suitable blocking molecules include recombinant pleiotrophin-receptor proteins of the invention, analogs of pleiotrophin-receptor proteins that retain pleiotrophin binding ability, but lack or have a modified tyrosine kinase activity, a transmembrane portion, insulin binding sites, transcription or translation controlling sequences, or any combination thereof. It is especially important to note that blocking may both inactivate the receptor or activate the receptor protein. Stimulation of the receptor is desired in instances where pleiotrophin activities are desired such as, for example, with non-cancerous endothelial cell proliferation for the development of blood vessels and surrounding tissues after ischemic attack.

The following examples illustrate embodiments of the invention, but should not be view as limiting the scope of the invention.

Examples

Example 1 Identification of the PTN receptor.

Signal transduction studies regarding the effects of PTN were impeded by the lack of a receptor involved. Recently, it was discovered that a receptor for PTN by phage display technique (Figure 1). PTN receptor fragments were identified using a phage display library of human fetal brain cDNAs. An M13 phage-display library of human fetal brain cDNA (EasyMATCH Phage

Display) was obtained from Clontech. The human cDNA fragments are located downstream of the phage gene III leader sequence in order to generate gene III fusion proteins that are exposed on the phage surface. Phage containing candidate PTN receptor cDNA fragments as inserts were selected by repeated panning of the library with purified PTN (~1 µg per well) that had been immobilized in the wells of a 96-well plate. Panning of selected clones against FGF-2 was used as a negative control.

Several rounds of panning against purified, biologically active PTN resulted in the isolation of two distinct phages that bound to PTN. Shown in Figure 1(a) is the general scheme for panning of a human fetal brain cDNA phage-display library against immobilized PTN protein. One of the selected clones contained an insert homologous to a sequence in the extracellular domain (ECD) of the orphan receptor ALK (GenBank number U66559). The regions and percent homology of the ALK protein with other tyrosine kinase receptors are indicated. NP-1 = neuropilin-1/2, LTK = leukocyte tyrosine kinase, IGF1-R = insulin-like growth factor-1 receptor, IR = insulin receptor, t(2,5) NPM ALK = nucleophosmin-ALK fusion protein. One of these encoded for a peptide sequence homologous to a region in the ECD of an orphan receptor tyrosine kinase, ALK (Stoica et al., 2001). Homology of the amino acid sequence in one of the phage inserts with a sequence stretch in the extracellular domain (ECD) of the Anaplastic Lymphoma Kinase (ALK) transmembrane receptor suggested that PTN could be a ligand for this orphan receptor with an apparent molecular mass of 200 to 220 kDa. As indicated in Figure 1a, the ligand binding domain recognized by the phage display is N-terminal of regions of homology between ALK and the leukocyte tyrosine kinase (LTK) – a close relative of ALK- and is not shared with any other protein in the data base (Stoica et al., 2001). ALK was discovered in anaplastic lymphoma and was found to be constitutively activated in this cancer due to a t(2,5) chromosomal translocation and a resulting fusion between the nucleophosmin protein and the intracytoplasmic kinase domain of ALK (Morris et al., 1994).

Example 2 Cell-free binding assays with nitrocellulose membrane-immobilized proteins.

To prove that the PTN ligand indeed binds to the ALK receptor, both cell-free and whole-cell assays were used. For the initial cell-free assays, the PTN protein or the recombinantly produced extracellular domain (ECD) of ALK or an aliquot of fetal calf serum were immobilized by spotting onto nitrocellulose membranes (Figure 1b). Briefly, PTN purified from the culture supernatant of cells transfected with the human PTN cDNA (approximately 5 ng of biologically active protein in

5 μ l) (Souttou et al., 1997) or the ECD of PTNR produced as an Fc fusion protein in CHO cells as described (Wen et al., 1992) (approximately 7.5 ng in 10 μ l), or fetal bovine serum as a negative control (2 μ l) were immobilized by spotting onto a nitrocellulose membrane. Nonspecific binding sites were blocked by incubation of the membranes with 5% (w/v) skim milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST). Membranes were then incubated for 2 hours at room temperature with PTN (in TBST), PTNR ECD protein (2 μ g/ml in TBST), or TBST alone (negative control) as appropriate, washed in TBST, and exposed overnight to rabbit antibodies to PTNR or mouse monoclonal antibodies to PTN (4B7). Antibodies were at a 1:20 dilution in PBS/0.1% Tween-20/1% BSA. As shown in Figure 1(b), binding of the PTN protein to the recombinant extracellular domain (ECD) of ALK. The PTN or the ALK ECD protein were immobilized on nitrocellulose membranes. After incubation without (-) or with (+) ALK ECD or PTN, respectively, bound PTN or ALK ECD were visualized by immunodetection with the respective antibodies. Bound antibody was visualized using commercially available reagents (Souttou et al., 1997; Souttou et al., 1998). Further details of the experiments are as described (Stoica et al., 2001).

In this assay anti-PTN antibodies recognized PTN bound to the immobilized ALK and, conversely, anti-ALK ECD antibodies recognized ALK that was bound to immobilized PTN. These data strongly support the notion that ALK and PTN can bind to each other. Still, it is possible that other molecules that are not detected by the immunodetection participate in this ligand/receptor interaction and are indeed major players. To alleviate this concern, an independent and alternative approach was used to monitor ALK binding and probed for the binding of PTN to its receptor by protein chip technology coupled to mass spectrometry.

Example 3 Protein chip technology to study PTN/receptor binding.

To obtain the most convincing data from the protein chip technology, ligand, PTN, was spiked into a mixture of proteins to render it barely detectable by mass spectrometry (Figure 1c; input). In this manner, it was reasoned that ligand could be recovered if the ligand/bait interaction is specific and of high affinity. This approach would also show whether other proteins bound to the immobilized ALK receptor at an equimolar or even higher amount than the proposed ligand, PTN. Analysis was performed with a SELDI (surface-enhanced laser desorption/ionization) (Dove, 1999) Protein Biology System I (Ciphergen, Palo Alto, CA). Different PTN-containing ligand preparations

(1 μ l of a 20 μ g/ml solution) were placed on a normal-phase protein array, which was then washed and 1 μ l of cyano-4-hydroxy cinnamic acid (2 mg/ml) in 50%(v/v) acetonitrile and 0.5%(w/v) trifluoroacetic acid was added to the spot. Retained proteins were then subjected to mass spectrometry. For the analysis of the interaction of PTN with PTNR, 3 μ l of a 240 μ g/ml solution of either the Fc PTNR ECD (see above) or the human TGF-beta receptor II (Sigma) in phosphate-buffered saline (PBS) were applied to a preactivated protein array, which was then incubated overnight in a humidified chamber at 4 °C. Protein solution was removed, 3 μ l of 1 M ethanolamine (pH 8.2) were added to each spot, and the array was incubated for an additional 30 min at room temperature. PTN (20 μ g/ml), either alone or in the presence of the PTNR ECD or TGF-beta receptor II each at a molar ratio of 1:1.7 relative to PTN, was dissolved in PBS containing 0.1% Triton X-100, and 3 μ l of each solution were added to the appropriate spots. After incubation of the chips for 1 hour at room temperature in a humidified chamber, each spot was washed three times with 5 μ l of PBS containing 0.1% Triton X-100, and the entire array was then washed once with 10 ml of 25%(v/v) ethylene glycol and twice with 10 ml of PBS. Proteins were then analyzed by mass spectrometry (Stoica et al., 2001).

As shown in Figure 1d, surface-enhanced laser desorption/ionization (SELDI) analysis (Dove, 1999) of PTN ligand binding to the ALK ECD was used. Mass spectrometry analysis of the “input” ligand preparation as well as of proteins bound to the immobilized ALK ECD or to a nonspecific protein control are shown. The arrowheads indicate the mass spectrometry position of the peak corresponding to the PTN protein. Proteins present in conditioned media (“input”) bound to immobilized TGF-b RII (nonspecific) or to the immobilized ALK ECD. After subtraction of nonspecific binding only the peak indicated by the arrowhead remained (Figure 1d). The ligand preparation (“input”, upper panel) was incubated with the TGF-b RII protein as a control competitor (middle panel) or with the ALK ECD as a specific competitor (lower panel) and then allowed to bind to the immobilized ALK ECD (Figure 1d). Analysis of bound proteins after SELDI shows that PTN binds to the immobilized ALK ECD and that this binding is competed by pre-incubation of the ligand solution with the ALK ECD.

The only specifically bound protein isolated from this was PTN (compare Figure 2a: nonspecific versus ALK ECD binding). As a further series of control experiments a more purified

PTN preparation (input) was used and showed a mass of 15,868 daltons for PTN. This mass was indistinguishable from the mass of the bound ligand obtained from binding studies to the ALK ECD using different PTN preparations (15882 ± 43 , $n=4$). Finally, it was also demonstrated that PTN binding to the immobilized ALK ECD was competed specifically by the presence of soluble ALK ECD in the incubation mixture (Figure 2b). As a control, an unrelated recombinant receptor protein (TGF-beta receptor II) was unable to compete.

Example 4 PTN receptor binding studies in intact cells.

Beyond the binding studies in the cell-free system, ligand receptor binding was studied in cultured cells to prove that the interaction also occurred *in vivo*. For this, ALK-negative mouse hematopoietic cell line 32D was used which grows in suspension culture and expressed the ALK protein in these cells. Also used was ^{35}S -labeled PTN from metabolic labeling experiments as a ligand for the receptor binding studies. Briefly, cell lysates were then prepared and a total of 3 mg of cellular proteins subjected to immunoprecipitation as described. (Souttou et al., 1997). For uncoupled antibodies, Sepharose-bound protein G (Gammabind plus, Amersham/Pharmacia) was used to precipitate immunocomplexes. Antibodies were anti-phosphotyrosine (agarose-coupled 4G10, 30 μl ; UBI, Lake Placid NY), anti-PTNR (a cocktail of antibodies from Santa Cruz Biotechnology, Santa Cruz CA and from Accurate Chemicals, Westburg NY), anti-IRS-1 (3 μg of rabbit IgG). Resulting precipitates were analyzed by SDS polyacrylamide gel electrophoresis. Experimental procedures for immunoblots for phosphotyrosine, IRS-1 (anti-IRS-1, Transduction Laboratories, Lexington KY), PLC-gamma (anti-PLC-gamma, UBI) and PI3-kinase (UBI) using unlabelled cells as described (Souttou et al., 1997).

As shown in Figure 2, competition for the binding of radiolabeled PTN (1 ng/ml) to 32D/ALK-transfected cells by cold PTN (30X), ALK ECD protein (0.7 $\mu\text{g/ml}$) or an affinity-purified anti-PTN antibody (2.5 $\mu\text{g/ml}$) or an IgG raised against an ECD fragment containing the ligand binding domain (6 $\mu\text{g/ml}$) (Figure 2a). Saturation binding of radiolabeled PTN to 32D/ALK (filled symbols) and 32D/control cells (open symbols) (Figure 2b). Data were pooled from two independent experiments with triplicate repeat measurements of data points. Fitted curves were obtained from non-linear regression analysis for saturation binding studies (Prism-Graphpad). A K_d -value of 32 ± 9 pM was calculated from this. Inset: Scatchard transformation of the binding data. B

is specific binding, i.e. the difference between binding to 32D/ALK and 32D/control cells; F refers to the free concentration of PTN.

These studies showed saturable binding to the cells when they expressed the ALK cDNA and only non-specific, linearly increasing binding without the receptor present (Figure 2b). Quantitation of these binding studies yielded an apparent dissociation constant of 30 to 40 pM, in close agreement with the effective concentrations of PTN in biological assays, i.e. in the 0.5 to 1 ng/ml range (Fang et al., 1992; Wellstein et al., 1992). Finally, in support of the specificity of the ligand / receptor interaction, PTN receptor binding was competed by anti-PTN and anti-ALK ECD antibodies as well as by added recombinant ALK ECD protein and unlabeled PTN (Figure 3a). In summary, these data confirm that the ALK ECD recognizes and binds PTN as a ligand also in intact cells.

Example 5 PTN signal transduction through the ALK receptor.

ALK receptor protein was over expressed in COS-7 and in SW-13 cells to study signal transduction. Sf-9 *Drosophila melanogaster* cells were cultured in EX-Cell 400 media (JRH Bioscience, Lenexa, KS), supplemented with 5% (v/v) fetal calf serum in a humidified incubator at 27°C in the absence of CO₂. SW-13 cells have been used in a number of studies with PTN (Fang et al., 1992; Souttou et al., 1998; Wellstein et al., 1992). Stable transfection of SW-13 human adrenal carcinoma cells (Wellstein et al., 1992) was performed by electroporation. Further details are described in (Stoica et al., 2001). As shown in Figure 3, ALK-transfected or control cells were grown in the presence of ³²P-orthophosphate and stimulated with PTN. Protein extract was immunoprecipitated with different antibodies, separated by SDS-PAGE and the phosphoproteins were detected by autoradiography. Transient transfection of COS-7 cells stimulated with PTN without (+) and with fetal calf serum (++) for 5 min and immunoprecipitation with an anti-phosphotyrosine antibody (Figure 3a). Immunoblot analysis for ALK showed equal expression in the ALK-transfected cells (not shown). Control SW-13 cells or SW-13/ALK stably transfected cells were stimulated with PTN for different time intervals as indicated and proteins immunoprecipitated with anti phosphotyrosine, anti-ALK or anti-IRS-1 antibodies (Figure 3b). Effect of addition of the ALK ECD-Fc fusion protein (0.7 mg/ml) or an affinity-purified anti-PTN antibody (2.5 µg/ml) on PTN-induced tyrosine phosphorylation in SW-13/ALK cells (Figure 3c). As under (Figure 3b) SW-13/ALK cells were stimulated for 5 minutes with PTN that had been pre-incubated with anti-PTN

or with the ALK ECD. The anti-phosphotyrosine Western blot (WB anti-PY) of immunoprecipitates with an anti-PY antibody (IP anti-PY) is shown.

Upon interaction of ALK with PTN, a series of proteins were found to be phosphorylated (Figures 3a, 3c). Phosphorylation was inhibited by the soluble ALK ECD and by antibodies against PTN (Figure 3d). Key signal transduction molecules phosphorylated are IRS-1 (Figure 3b), PLC-gamma, PI3-kinase and Shc (Stoica et al., 2001).

Example 6 ALK expression in human tumor cell lines.

Pleiotrophin is expressed at detectable levels in the normal adult nervous system and over expressed in brain tumors as well as in other cancers (Schulte et al., 1997). To evaluate the potential contribution of pleiotrophin signaling, expression of its receptor, ALK, in human glioblastoma samples and in tumor cell lines was examined. A dose-response relationship for the effect of PTN on colony formation in soft agar by SW-13/control and SW-13/ALK cells was determined. One representative of three independent experiments with mean \pm S.E. of triplicate dishes is shown in Figure 4. Inset: Western blot analysis for ALK protein in lysates of ALK-transfected or control SW-13 cells. For the tumor samples, immunohistochemistry was used to identify the ALK protein and assessed the expression relative to normal brain. Shown in Figure 5 is ALK expression in human glioblastoma (GBM) tissue and cell lines. Detection of ALK protein in human glioblastoma tumor samples by immunohistochemistry (Figures 5a-d). Adjacent normal brain is negative for ALK protein by immunohistochemistry. The space bar equals 20 μ m (Figure 5e). Detection of human ALK mRNA in cell lines by RNase protection. Three of seven human glial cell lines (U87MG, U118MG and U138MG) are positive for ALK mRNA. C6 rat glioma and SW-13/mALK express rodent ALK mRNA and should not show cross-reactivity with the human ALK probe. 36B4 is a loading control.

Glioblastoma multiforme show a strong staining for ALK, whereas the signal was below detection in normal adjacent brain tissues (Figure 5). This was confirmed in parallel experiments with four additional cases of glioblastoma (not shown). Anti-ALK antibodies raised against different antigens, i.e. the extracellular domain (ECD) of ALK and ECD-derived peptides gave the same distinctive result (not shown). In cultured cell lines, ALK mRNA was found expressed in three of seven human glioma or glioblastoma lines by RNase protection. A control for the specificity of the

human ALK probe shows no cross-reaction with rodent ALK mRNA (C6 and SW-13/murine ALK-transfected cells; Figure 5e). Furthermore, pleiotrophin mRNA was found to be expressed in the ALK-positive cell lines (not shown) supporting a role of pleiotrophin as an autocrine as well as a paracrine stimulator.

5 Example 7 PTN signal transduction in glioblastoma cells via P13-kinase.

The U87MG cell line was used to study the contribution of the pleiotrophin-ALK axis to the malignant phenotype of glioblastoma. U87MG cells are a well-characterized model system to study tumorigenesis and signaling in glioblastoma (Li et al., 1998; Wen et al., 2001) and express different receptor tyrosine kinases and their ligands such as EGF-R (O'Rourke et al., 1997) and PDGF-R (Nister et al., 1991). Furthermore, U87MG cells contain a mutated and inactive form of the tumor suppressor PTEN (exon 3) (Furnari et al., 1997; Maehama et al., 1999) and expression of an active form of PTEN suppresses their tumorigenicity and tumor angiogenesis (Li et al., 1998; Wen et al., 2001). The lipid phosphatase PTEN controls signaling pathways that involve PI3-kinase activity (Maehama et al., 1999) and was initially examined for pleiotrophin signals through PI3-kinase in the U87MG cells. For this phosphorylation of the downstream target molecule, Akt, was monitored an anti-apoptotic effector of PI3-kinase signaling that is activated by phosphorylation (Maehama et al., 1999; Khwaja, 1999). As shown in Figure 6, pleiotrophin-induced Akt phosphorylation in U87MG cells. Representative immunoblots of the effects of pleiotrophin on Akt phosphorylation (on serine 473) in serum-starved wildtype U87MG cells: (Figure 6a) Dose-response of pleiotrophin on Akt phosphorylation. (Figure 6b) Time-course following treatment with 2 ng/ml pleiotrophin. (Figure 6c) Inhibition by LY294002 and wortmannin of Akt phosphorylation in response to treatment with 1 ng/ml pleiotrophin. Akt protein blots are shown in the respective lower panels. Pleiotrophin induced Akt phosphorylation at Serine 473 in a dose-dependent manner and reached saturation at a very low concentration of the ligand (<1 ng/ml) after five minutes of treatment (Figure 6a). The phosphorylation signal was maintained for at least one hour (Figure 6b). The rapid response suggested that Akt phosphorylation is not due to a secondary effect of pleiotrophin signaling but rather due to a close link between pleiotrophin's activated receptor and PI3-kinase. Pleiotrophin-induced Akt phosphorylation was inhibited by pretreatment of U87MG cells with the inhibitors LY294002 and wortmannin, confirming that this effect is indeed mediated by PI3-kinase (Figure 6c).

Interestingly, the MAP kinase pathway in the U87MG cells is activated constitutively and no increase in phosphorylation was observed after treatment with pleiotrophin (not shown). This suggested that the glioblastoma cells predominantly use the PI3-kinase pathway for pleiotrophin-ALK signaling in contrast to epithelial cells in which ALK stimulation by pleiotrophin results in activation of both the MAPK and PI3-kinase pathways (Souttou et al., 1997; Stoica et al., 2001).

Example 8 Ribozyme-mediated reduction of endogenous ALK reduces PTN signaling.

To assess whether pleiotrophin signaling through ALK is rate-limiting for the malignant phenotype of U87MG cells, derivative cell lines were generated in which the endogenous ALK is reduced by constitutive expression of ALK mRNA targeted ribozymes. Shown in Figure 7 is the effect of ALK depletion by ribozymes on pleiotrophin signaling. Detection of ALK mRNA by RNase protection assay in empty vector (pRC/CMV) and different ribozyme transfected U87MG cells. Autoradiogram (Figure 7a) and quantitation by phosphoimager analysis (Figure 7b); are shown relative to control (pRC/CMV). Dose-response of pleiotrophin (Figure 7c) and PDGF BB (Figure 7d) on pRC/CMV cells (squares), Rz1-7 cells (triangles) and Rz2-2 cells (circles). Comparison of the effect of pleiotrophin (0.5 ng/ml; open circles) or PDGF BB (20 ng/ml, closed circles) as a function of ALK levels in the different cell lines from panel (b) (Figure 7e). Densitometric analyses of results of the immunoblots are presented as the mean of three independent experiments +/- S.E.M.

This approach has been applied to evaluate the contribution of different gene products to the malignant phenotype (Czubayko et al., 1994; Czubayko et al., 1996; Schulte et al., 1996; Czubayko et al., 1997; Wellstein et al., 1999) and initially screened different ALK-targeted ribozymes for their efficacy. From this, two ribozyme expression vectors were selected that target sequences in the 5' and in the 3' ends of the ALK mRNA (Rz1 and Rz2 respectively) and generated a panel of stably transfected U87MG cell lines with high, medium and low residual ALK mRNA levels (Figure 7a and b). In these cell lines the ability of pleiotrophin to stimulate Akt phosphorylation was reduced in parallel with the reduced endogenous ALK mRNA (Figures 7c and e). In contrast, Akt phosphorylation induced through an independent tyrosine kinase receptor (PDGF-R) that is expressed in U87MG cells (Nister et al., 1991) was unaffected by the reduction of ALK (Figure 7d

and e). From these results, it was concluded that pleiotrophin's ability to induce Akt phosphorylation is strictly dependent on ALK.

Example 9 Reduction of ALK and tumor growth and animal survival.

Interestingly, the derivative U87MG cells with different residual levels of ALK showed no significant difference in their proliferation rate or colony formation in soft agar (not shown). This suggests that under *in vitro* growth conditions, ALK is not a rate-limiting factor. To determine if the reduction of pleiotrophin-ALK signaling affects the *in vivo* tumor growth of the U87MG cells, the different cell lines were grown as tumor xenografts in nude mice. As shown in Figure 8, xenograft tumor growth (a) size of pRC/CMV (squares), Rz1-2 (diamonds), Rz1-7 (triangles) and Rz2-9 (circles) xenograft tumors as a function of time after tumor cell inoculation.

Tumor size of different cell lines at day thirteen as a function of relative ALK levels (Figure 8a), and survival curves of mice from panel (a). Control cells with the highest residual levels of ALK formed rapidly growing tumors (Figure 8a) at a rate that was indistinguishable from wild-type cells (not shown). In contrast, tumor growth of the ALK-depleted cells was significantly reduced (Figure 8a). As with the responsiveness to pleiotrophin induction of Akt phosphorylation, the tumor size of the xenografts grown from the different cell lines was directly dependent on the level of ALK expression in a "gene-dose"-dependent manner (Figure 8b). This "gene-dose" effect was observed with clonal and mass-transfected cell lines (not shown). Finally, the difference in tumor growth also resulted in a shift of the survival curve: while all of the control mice died by the twentieth day after injection, most mice injected with the ALK-depleted cells survived at least twice as long (Figure 8c) and the median survival correlated with the residual ALK levels.

Example 10 ALK levels and mitotic index and apoptosis in tumors.

In light of the fact that pleiotrophin signaling through ALK activates a pathway known to promote cell survival, namely PI3-kinase and Akt, it was decided to investigate the rate of apoptosis in the xenograft tumor specimens and compare this to the mitotic index in the same samples. Using sections from size-matched tumors, H&E and TUNEL stained sections were examined for mitotic and apoptotic cells respectively (Figure 9a). Analysis of mitosis and apoptosis in tumor xenografts using (a) a high power (400 x) H & E (left column) and TUNEL

(right column) stained sections from size-matched pRC/CMV (upper row) and Rz1-2 (lower row) xenograft tumors, showing (b) the number of mitotic figures (open circles) and TUNEL positive cells (closed circles) as a function of relative ALK levels. Results are presented as the mean of twenty high power (400 x) fields +/- S.E.M.

5 No significant difference was found in the number of mitotic figures but a striking difference in the numbers of TUNEL positive cells. In fact, the number of TUNEL positive cells correlated directly with the reduction of ALK levels (Figure 9b). Recent studies suggested a role of PTEN in tumor angiogenesis (Wen et al., 2001) and thus it was also assessed whether the reduction of ALK in the U87MG cells influenced the *in vitro* production of endothelial cell growth factors as well as
10 the extent of tumor angiogenesis *in vivo*. Both, the endothelial cell growth-stimulatory activity present in the supernatants of the different U87MG cell lines as well as the extent of tumor angiogenesis measured in the tumor samples was not significantly affected by the reduction of ALK (not shown). Overall, these findings indicate that the pleiotrophin-ALK signaling provides an essential survival signal that is rate-limiting for tumor growth of U87MG cells.

5 Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. All references cited herein, including all publications, U.S. and foreign patents and patent applications including U.S. provisional application number 60/211,491 entitled "Receptor for the Growth Factor PTN," filed June 14, 2000, are specifically and entirely hereby incorporated herein by reference. It is intended
20 that the specification and examples be considered exemplary only, with the true scope and spirit of the invention indicated by the following claims.